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Betaine or folate can equally furnish remethylation to methionine and increase transmethylation in methionine-restricted neonates

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Abbreviations used: folate, 5-methyl-tetrahydrofolate; BHMT, betaine:homocysteine methyltransferase; CBS, cystathionine beta-synthase; DMG, dimethylglycine; FPF, fraction of product flux; FNC, fractional net conversion; Hcy, homocysteine; **MD-**, methyl-deplete diet; MSyn, methionine synthase; **MS+F**, methyl-deplete diet plus folate; **MS+B**, methyl-deplete diet plus betaine; **MS+FB**, methyl-deplete diet plus folate and betaine; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine methyltransferase; RM, remethylation; Q, flux; PS, protein synthesis; PB, protein breakdown; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TM, transmethylation; TS, transsulfuration

Abstract

Methionine partitioning between protein turnover and a considerable pool of transmethylation precursors is a critical process in the neonate. Transmethylation yields homocysteine, which is either oxidized to cysteine (*ie*, transsulfuration), or is remethylated to methionine by folate- or betaine- (from choline) mediated remethylation pathways. The present investigation quantifies the individual and synergistic importance of folate and betaine for methionine partitioning in neonates. To minimize whole body remethylation, 4-8-d-old piglets were orally fed an otherwise complete diet without remethylation precursors folate, betaine and choline (i.e. methyl-deplete, **MD-**) (n=18). Dietary methionine was reduced from 0.3 to 0.2 g/(kg·d) on day-5 to limit methionine availability, and methionine kinetics were assessed during a gastric infusion of [$^{13}C_1$]methionine and [$^{2}H_3$-methyl]methionine. Methionine kinetics were reevaluated 2 d after pigs were rescued with either dietary folate (38 µg/(kg·d)) (**MD+F**) (n=6), betaine (235 mg/(kg·d)) (**MD+B**) (n=6) or folate and betaine (**MD+FB**) (n=6). Plasma choline, betaine, dimethylglycine (DMG), folate and cysteine were all diminished or undetectable after 7 d of methyl restriction (P<0.05). Post-rescue, plasma betaine and folate concentrations responded to their provision, and homocysteine and glycine concentrations were lower (P<0.05). Post-rescue, remethylation and transmethylation rates were ~70-80% higher (P<0.05), and protein breakdown was spared by 27% (P<0.05). However, rescue did not affect transsulfuration (oxidation), plasma methionine, protein synthesis or protein deposition (P>0.05). There were no differences among rescue treatments; thus betaine was as effective as folate at furnishing remethylation.

Supplemental betaine or folate can furnish the transmethylation requirement during acute protein restriction in the neonate.

1. Introduction

The metabolic demands of protein turnover and transmethylation are integral to the methionine requirement, and partitioning among these critical processes ensures that growth and maintenance demands are fulfilled in the neonate. However, quantifying methionine partitioning is complicated because methyl donors in the form of 5-methyl tetrahydrofolate (folate) and betaine (derived from choline) serve to remethylate homocysteine (*ie*, demethylated methionine) and re-form methionine (**Supplemental Figure 1**). The capacity of remethylation flux to furnish transmethylation and protein turnover has been demonstrated in a methionine-restricted neonatal piglet model [1,2], but it is yet unclear whether dietary remethylation precursors must act in synergy, or whether betaine/choline or folate can sufficiently supply remethylation demands independently.

The enzymes that facilitate remethylation from folate and betaine are methionine synthase (MSyn) and betaine:homocysteine methyltransferase (BHMT), respectively. Interestingly, BHMT and MSyn appear to be both interrelated and adaptable. For example, a low choline intake in rats leads to depleted hepatic folate concentrations [3], whereas a low folate intake depletes hepatic choline concentrations [4]. However, in the latter case, it is unknown whether choline depletion was due to greater consumption (via greater BHMT activity) or insufficient production (via lower synthesis from phosphatidylcholine (PC)). The production of choline from PC oxidation is a major metabolic process [5], and the further oxidation of choline to betaine allows the body to

reform methionine, which in turn can be used to synthesize PC via transmethylation. Due to the nature of choline as both a transmethylation product (via phosphatidylethanolamine methyltransferase (PEMT)) and remethylation precursor (via betaine), its supplementation could potentially downregulate transmethylation as well as enhance remethylation. However, choline is not considered a labile methyl donor, *per se*, since betaine is the actual precursor for BHMT. The focus of this investigation was to determine whether betaine or folate can furnish remethylation equally.

Studies that have compared supplementation with remethylation precursors, folate and betaine, have provided conflicting, and sometimes confusing results. For example, in broiler hens fed graded levels of methionine, choline and betaine, the majority of *in vitro* remethylation was attributed to MSyn [6]. Similarly, in otherwise well-nourished young women, remethylation via MSyn accounted for the vast majority of total remethylation, and although folate restriction marginally reduced the percent of remethylation from serine (*ie,* via the folate cycle), it had no effect on whole body remethylation; the authors suggested that "a one-carbon donor other than serine (e.g. betaine) can be used to compensate for impaired folate-dependent remethylation" [7]. Indeed, betaine supplementation did not increase remethylation in folate-replete young men, but transmethylation and methionine oxidation were both greater. It was suggested by those investigators that excess betaine may *increase* the methionine requirement [8]. And finally, betaine and folate supplementation were both effective at reducing circulating homocysteine; however, betaine supplementation was considered more effective at mitigating a homocysteine rise during a methionine load test [9]. What these studies collectively demonstrate is that both betaine and folate are responsive to higher

remethylation demand, but whole body remethylation is tightly regulated under conditions of adequate dietary methyl supply. Our group has recently shown that remethylation is highly responsive to the presence of choline, betaine and folate during methionine restriction *in vivo* [1], but it is not clear whether folate or betaine can fulfill *in vivo* remethylation demands alone.

The objective of this study was to measure the effects of folate and betaine on methionine partitioning in a methyl-restricted neonatal piglet. Methionine partitioning was measured initially in piglets fed a diet devoid of folate, choline and betaine, and was reassessed after the provision of folate, betaine or folate + betaine. The subsequent provision of dietary remethylation precursors was termed rescue, and is used hereafter to refer to the period after the repletion of a dietary remethylation precursor(s).

2. Materials and Methods

2.1. Chemical reagents and isotopes

All chemicals and reagents were of the highest available purity and were obtained from Sigma (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ) or Alfa Aesar (Ward Hill, MA). Amino acids were from Ajinomoto, Co (Tokyo, Japan). [^{14}C]methyl-tetrahydrofolic acid ([^{14}C]MTHF), barium salt was from Amersham Biosciences, UK Limited (Buckinghamshire, UK). L-[3-^{14}C]Serine ([^{14}C]serine) and [^{14}C-methyl]-N,N,N-trimethyl glycine, ([^{14}C]betaine) were acquired from Moravek Biochemicals (Brea, CA). DL-[3,3,3',3',4,4,4',4'-^{2}H]homocystine ([^{2}H]homocystine) (internal standard), L-[$^{13}C_1$]methionine ([^{13}C]methionine) and L-[$^{2}H_3$-methyl]methionine were obtained from Cambridge Isotope Laboratories (Tewksbury, MA).

2.2. Piglets and surgical procedures

The animal care committee at Memorial University of Newfoundland approved all animal protocols performed herein. Eighteen 4-8-d-old Yucatan miniature piglets (9 male, 9 female) were removed from the sow and transported to a small animal care facility from the University vivarium. Animals were anesthetized with intramuscular injections of acepromazine (0.5 mg/kg; Atravet; Ayerst Laboratories), ketamine hydrochloride (20 mg/kg; Rogarsetic Rogar STB) and atropine (20 mg/kg; Rafter Dex Canada). An endotracheal tube was inserted and animals were maintained on anesthesia using 0.5-1.0% isoflurane. Gastric and venous catheters were surgically implanted as previously described [10].

2.3. Dietary regimens and study protocol

Piglets were fed elemental diets continuously using a regimen described previously [1,2,10-12]. Briefly, piglets were adapted to gastric diets post-surgery and within 36 h were fed 100% of target rate of 11.3 mL/(kg·h). Both adaptation and test diets provided 16 g/(kg·d) of protein supplied by crystalline amino acids and 1.1 MJ/(kg·d) of non-protein energy was provided in a 1:1 ratio of carbohydrate (dextrose) and fat (soybean oil); oil was administered into the diet line at a rate of 0.12 mL/(kg·h). Vitamins and minerals were provided in the diet at >100% of piglet requirements. Pigs were weighed daily and diet infusion rates adjusted accordingly.

Piglets (n = 18) were fed adaptation diets that were devoid of dietary folate, betaine and choline, but were otherwise nutritionally replete for the first 5 d, as described

previously [11]. These experimental dietary conditions were used to minimize whole body remethylation by omitting remethylation pathway precursors; the effects of dietary remethylation precursors can then be assessed by re-introduction. Although serine and glycine are also considered methyl donors, because they are non-essential amino acids and readily synthesized, they were not omitted from the diet. On the evening of study Day-5, the dietary methionine supply changed from 0.3 mg/(kg·d) to 0.2 mg/(kg·d) (**MD-**), in order to restrict available methionine to approximately 80% of the piglet methionine requirement [10-12], which would allow detection of an increase in methionine availability due to remethylation; only 16 h of dietary adaptation are necessary to elicit a response to methionine availability without compromising health [12]. The **MD-** piglets were fed this diet until study Day-7 (*ie*, Pre-Rescue) when they received a primed:constant infusion (20 µmol/kg:10 µmol/(kg·h), respectively) of [^{13}C]methionine and [$^{2}H_3$-methyl]methionine into their gastric catheter for 8 h. The infusion occurred in an airtight plexiglass chamber that permitted frequent blood sampling through the jugular catheter as well as breath sampling and collection using a metabolic cart system (Qubit Systems, ON) [1,11]. Immediately following the infusion, piglets were fed the same **MD-** diet supplemented with 38 µg/(kg·d) folate (**MD+F**) (n = 6), 235 mg/(kg·d) betaine (**MD+B**) (n = 6) or both folate and betaine (**MD+FB**) (n = 6). Animals remained on these 'rescue' diets until the completion of the repeated methionine infusion on Day-10 (*ie*, Pre-Rescue). Because neonatal methionine metabolism adapts to dietary intake changes within 16 h [12], we expected a 2-day rescue to be sufficient to change remethylation to methionine in a steady state manner. To account for dietary contribution of methionine tracers, methionine in the diets during isotope infusion was reduced so total methionine

intake was unchanged. Folate was supplemented at a rate providing 200% of the daily folate requirement in a piglet and betaine was supplemented at the molar equivalent of the methionine requirement [11]. At the end of Day-10, piglets were anesthetized and their livers were rapidly extracted and flash frozen. Liver samples were stored at -80 °C until used to measure cystathionine beta-synthase (CBS), MSyn and BHMT activities. A schematic representation of the study design is found in **Figure 1**.

2.4. Analytical Procedures

Plasma amino acids were measured with their phenylisothiocyanate derivatives as described previously [13]. Methionine kinetics were calculated by measuring plasma enrichment with a GCMS in EI mode following derivatization with pentafluorobenzyl bromide and by monitoring the following ions: 328 for methionine, 329 for [$^{13}C_1$]methionine, 331 for [$^{2}H_3$-methyl]methionine, 494 for homocysteine, and 495 for [$^{13}C_1$]homocysteine [1]. Choline, betaine and dimethylglycine concentrations (and kinetics) were measured by chromatography on an Atlantis HILIC Silica 3 µm, 2.1 x 100 mm column and a Waters Alliance 2795 HPLC system (Waters Corporation) tandem mass spectrometry on a Micromass Quattro Ultima tandem mass spectrometer (Waters Corporation). Compounds were analyzed in multiple-reaction monitoring mode using the following transitions for the corresponding M+1 ion in each case: [$^{2}H_3$]betaine 121 → 62, betaine 118 → 59, [$^{2}H_3$-methyl]choline 107 → 63, choline 104 → 60, [$^{2}H_3$]dimethylglycine 104 → 58, and dimethylglycine 101 → 55. Choline and betaine concentrations were quantified using internal standards and the following transitions: [$^{2}H_{11}$]betaine 129 → 68 and [$^{2}H_9$-methyl]choline 113 → 69 [1,14]. The collection and

enrichment of [$^{13}CO_2$] in breath was measured using an IRMS as described previously [11]. Briefly, piglets were infused with isotopes while in air-tight chambers with air drawn through the boxes with a vacuum pump. Breath samples with [$^{13}CO_2$] were collected in 1 M NaOH and the rate of CO_2 production determined using an indirect calorimetry system (Qubit Systems). The *in vitro* activities of MSyn, BHMT and CBS were measured in liver homogenates exposed to radioactive substrates as reported elsewhere [1,15].

2.5. Calculations

All calculations have been reported previously [1]. Briefly, whole body methionine flux was calculated using isotope dilution in plasma. The kinetic parameters of methionine metabolism, *ie*, transmethylation, transsulfuration and remethylation, were calculated by: the difference between [$^{13}C_1$]methionine and [$^{2}H_3$-methyl]methionine flux rates (*ie*, transmethylation); the appearance of [$^{13}CO_2$] in breath (*ie*, transsulfuration); and the difference between transmethylation and transsulfuration (*ie*, remethylation) [1,16]. Plasma [M+1]homocysteine enrichment was used to reflect intracellular methionine enrichment [17]. Choline enrichment in plasma was used to calculate the fraction of methionine flux that was devoted to choline oxidation by the fraction of product flux ($FPF_{Methionine \rightarrow Choline}$) and fractional net conversion ($FNC_{Methionine \rightarrow Choline}$), as described previously [1].

2.6. Statistics

Paired t-test was used to compare piglet weights, plasma concentrations of folate, choline, betaine and DMG between Day-0 and Day-7 as well as plasma concentrations of methionine, cysteine and homocysteine between Day-1 and Day-7. Methionine kinetics were analyzed by two-way ANOVA with rescue (Pre-Rescue on Day-7 versus Post-Rescue on Day-10) and dietary remethylation precursor (MD+F, MD+B, MD+FB) as main effects; when the interaction was significant, group means were compared using a Bonferroni post-test. Enzyme activities and DMG concentration were compared by one-way ANOVA and Tukey's post-hoc test on Day-10. All statistics were calculated using Prism software 5.0b (La Jolla, CA). A *P*-value of < 0.05 was considered significant in all cases. Values are presented as mean ± standard deviation.

3. Results

3.1. Animals

All groups were balanced for weight and sex. Pre-rescue piglet weights were 2.4 ± 0.3, 2.4 ± 0.4 and 2.4 ± 0.2 kg for folate, betaine, and folate + betaine, respectively, and were higher post-rescue (2.9 ± 0.1, 2.9 ± 0.4 and 2.7 ± 0.3 kg for folate, betaine, and folate + betaine, respectively), with no differences in weight among supplementation groups. All animals gained weight during methyl restriction and there was no effect of any particular methyl rescue group on fractional weight gain between Day-0 and Day-7 (91.0 ± 27.4, 93.9 ± 69.9, and 77.5 ± 20.6 g/(kg·d) for folate, betaine, and folate + betaine, respectively) or between Day-7 and Day-10 (85.6 ± 48.1, 71.1 ± 16.2, and 66.9 ± 36.5 for folate, betaine, and folate + betaine, respectively).

3.2. Effects of methyl restriction on plasma metabolites

The effect of methyl restriction on methionine cycle intermediates was assessed by comparison of plasma metabolite concentrations at baseline (Day-0 or -1) and Pre-Rescue on Day-7 (**Table 1**). Even with this short 7-day intervention, plasma choline, folate and DMG concentrations decreased by 70%, 65% and 99%, respectively ($P < 0.05$) during **MD-** feeding. Plasma betaine concentration dropped below the limit of detection by Day-7 in **MD-** piglets. The concentration of plasma homocysteine was unchanged after **MD-** feeding but plasma methionine and cysteine concentrations dropped 30% ($P = 0.0004$) and 45% ($P < 0.0001$), respectively, during this same period. The validation of feeding this methyl-restricted diet in rapidly growing piglets has been described previously [1,11].

3.3. Effect of rescue on plasma metabolites

The effects of methyl rescue on methionine cycle intermediates and relevant amino acids are summarized in **Table 2**. Folate was above the analytical measurement range (>80 ng/mL) in the **MD+F** and **MD+FB** piglets Post-Rescue. Betaine was below the limit of detection (<0.55 μM) during **MD-** feeding on Day-7 (Pre-Rescue) and in the **MD+F** piglets Post-Rescue. This depletion of betaine was supported by the low circulating DMG concentrations Post-Rescue in **MD+F** piglets. There was no interaction among individual rescue groups on the other circulating metabolites, and so data from all groups were pooled on Day-10 (**MD+Rescue**) and were compared to **MD-** feeding on Day-7 using paired t-test. Plasma homocysteine concentration was 33% lower (30.3 ±

14.1 **MD-** *versus* 21.0 ± 11.0 **MD+Rescue**; $P = 0.001$) and glycine was 22% lower with rescue (137.7 ± 33.7 **MD-** *versus* 107.3 ± 32.4 **MD+Rescue**; $P = 0.009$), which respectively suggest enhanced remethylation and transmethylation. Plasma methionine, choline, cysteine, serine, arginine and taurine concentrations were unaffected by the provision of betaine and folate after methyl restriction ($P > 0.05$).

3.4. Methionine metabolism

The enrichments of [$^{13}C_1$]methionine, [$^{2}H_3$-methyl]methionine, [$^{13}C_1$]homocysteine and [$^{2}H_3$-methyl]choline were at plateau in plasma and breath by 6 h (**Figure 2)**. The error bars indicate the standard deviation of enrichment measures from all piglets. Steady state [$^{2}H_3$-methyl] enrichment was not achieved for betaine nor its demethylated product DMG. Remethylation data from one **MD+B** piglet were excluded as outlier due to very high variability (>3 SD from mean). The flux of the two methionine tracers were unaffected by methyl group rescue, but the feeding of any remethylation precursor increased remethylation ($P < 0.0001$) and transmethylation rates ($P < 0.0001$) (**Table 3, Figure 3**); the increases in remethylation ($P = 0.58$) and transmethylation ($P = 0.52$) were not different among methyl group treatments. When treatment groups were pooled, remethylation increased by 80% from 8.5 ± 5.0 μmol met/(kg·h) Pre-Rescue (**MD-**) to 15.4 ± 6.7 μmol met/(kg·h) Post-Rescue on Day-10 (**MD+Rescue**) ($P < 0.0001$). Moreover, transmethylation increased by 70% from 9.5 ± 5.5 μmol met/(kg·h) Pre-Rescue on Day-7 (**MD-**) to 16.2 ± 6.8 μmol met/(kg·h) Post-Rescue on Day-10 (**MD+Rescue**) ($P < 0.00001$). Transsulfuration was unaffected by rescue and was low in

all pigs, which was anticipated due to the marginal methionine intake and presence of cysteine in all diets.

Whole body protein synthesis was unaffected by rescue based on two-way ANOVA; but the proportion of methionine flux for protein synthesis (*ie*, PS/Q_M) was reduced by methyl rescue ($P < 0.05$), concomitant with a higher proportion of flux for transmethylation (*ie*, TM/Q_M) ($P < 0.05$). As a result, methionine partitioning between protein synthesis and transmethylation (*ie*, PS/TM) was lower with rescue ($P < 0.05$) (**Table 3**). Methyl rescue also led to lower whole body protein breakdown ($P < 0.05$) but protein deposition was unchanged. In all cases the paired data matched significantly ($P < 0.0001$).

Dietary remethylation precursors had a profound effect on choline enrichment (MPE), which reflects enhanced PC oxidation to free choline (*via* methionine) when expressed as the fraction of product flux ($FPF_{Methionine \rightarrow Choline}$) ($P = 0.0007$) and fractional net conversion ($FNC_{Methionine \rightarrow Choline}$) ($P = 0.001$) (**Table 3**). There was no statistical interaction among individual remethylation precursors.

Hepatic MSyn, BHMT and CBS activities were not sensitive to rescue treatment (**Table 4**), which is in line with *in vivo* data demonstrating no interaction among treatment groups.

4. Discussion

The study objective was to determine the individual and synergistic effects of betaine and folate on remethylation and methionine partitioning to transmethylation or protein synthesis during the neonatal phase, when all metabolite pools are rapidly

expanding. To accomplish this objective, we developed a nutritional model to minimize remethylation by limiting dietary precursors for remethylation pathways. In order to detect an increase in methionine availability with methyl rescue, we also fed a moderately deficient methionine diet. The resulting change in methionine metabolism allowed us to quantify the contribution of remethylation to methionine availability, which will be applicable to a complete nutritional model. This model successfully reduces whole body remethylation flux by 60% [1]. After two days of rescue with dietary folate, betaine or both, we hypothesized that the increased remethylation will increase available methionine and stimulate protein synthesis and/or transmethylation pathways. We found folate and betaine to be equally capable of increasing methionine availability by remethylation, which translated to similar transmethylation flux. Furthermore, there appears to be no metabolic advantage of providing both folate and betaine, as betaine alone was as effective as folate in regenerating methionine for transmethylation.

Rescue with remethylation precursors resulted in higher remethylation, which was anticipated as homocysteine reduction has been shown to occur with folate [18] and betaine [9] supplementations in humans. Moreover, betaine was as effective as folate in facilitating whole body remethylation *in vivo*, and supplementation with both folate and betaine **(MD+FB)** did not further increase remethylation. Notably, the rescue by folate and betaine increased remethylation to rates (15.4 ± 6.7 μmol Met/(kg·h)) similar to those in piglets fed complete diets (with folate, choline and betaine) for 8 d (12.1 ± 6.0 μmol Met/(kg·h)) [1]. These data demonstrate fine control of remethylation in response to methyl group availability in neonates, which is in agreement with data in adults [7,8,19]. Moreover, by feeding an elemental diet to neonatal piglets and using isotope kinetics

approaches, we were able to calculate the contribution of remethylation to methionine availability. Combined, the provision of remethylation precursors led to 80% more *de novo* methionine, or + 6.9 µmol methionine/(kg·h). While plasma methionine concentration was unaffected by methyl rescue, the sum of remethylation and dietary methionine during **MD-** feeding was 96.5 µmol methionine/(kg·h), which increased to 103.4 µmol methionine/(kg·h) during rescue, and equals the dietary methionine requirement of the piglet [20]. However, the published requirement does not factor for methionine made available from remethylation, and these comparisons may not be valid.

Folate and/or betaine provision led to greater *in vivo* methionine partitioning towards transmethylation, and reduced protein breakdown (Figure 3). Indeed, methyl group rescue led to a greater fraction of methionine flux for transmethylation (*ie,* TM/Q_M), which corresponded with a reduced fraction of methionine flux for protein synthesis (*ie*, PS/Q_M). These data suggest that during low methionine availability, transmethylation is secondary to protein synthesis in neonates. Furthermore, protein breakdown was reduced post-rescue suggesting that during methyl restriction, methionine that was initially partitioned towards protein and then released during breakdown also tended to be made available for transmethylation. In other words, as methyl groups facilitated remethylation after rescue, then more methionine was available for partitioning through transmethylation, and the reliance on protein breakdown as a methionine source was reduced.

We found that the provision of a methyl group resulted in significantly higher *de novo* choline formation. This is in agreement with our previous finding that the FSR of PC was greater during **MD-** feeding [10]. Assuming that hepatic PEMT was also

enhanced in these **MD-** piglets, we hypothesize that the provision of remethylation precursors resulted in a higher flux through PEMT, which is supported by greater [M+3] choline appearance during rescue. The appearance of the [^{3}H-methyl] moiety into plasma choline was traced to gain insight into an important transmethylation pathway that can also feed remethylation. In a previous study, we found that feeding diets devoid of folate, betaine and choline led to greater flux through PEMT to synthesize PC and presumably provide choline [1,10]. In the current study using the same **MD-** diet, we have shown that adding back betaine and/or folate indeed led to enhanced choline production from PEMT flux. These data suggest that PEMT is sensitive to methyl supply and may regulate methyl balance through choline production. Because plasma choline did not change with rescue, the newly synthesized choline was either rapidly reincorporated into PC by the cytidine diphosphate-choline pathway to help overcome choline restriction [22], or it could have been used to synthesize betaine to reform methionine. Future investigations may quantify choline flux in these animals to gain further insight into the potential cycling of methyl groups through choline, as well as to quantify the partitioning of choline in tissues.

Methyl rescue did not have a discernible effect on *in vitro* remethylation or transsulfuration enzymes. This finding was contrary to the hypotheses that BHMT and MSyn activities would be diminished when their respective substrates were eliminated, or induced when the opposite substrate was eliminated [6,7]. Plasma methionine concentration was not affected by rescue, and because BHMT, MSyn and CBS activities have been shown to be governed by methionine concentration in rat liver slices [23,24], it is perhaps not surprising that in *vitro* capacity of these enzymes was not affected.

Furthermore, we have also previously reported that BHMT, MSyn and CBS activities and expressions were not sensitive to MD- feeding compared to methionine-restricted piglets fed choline, betaine and folate [1]. Interestingly, plasma folate concentration more than doubled after betaine rescue (Table 2), suggesting that betaine supplementation facilitated flux of methyl groups via both BHMT and MSyn, possibly via dimethylglycine which is a product of BHMT and can provide methyl groups for the folate pathway [6]. Similarly, folate rescue led to greater choline production (Table 3), which may have provided betaine for BHMT despite no detectable rise in plasma betaine in that group. Although plasma folate was 65% lower with MD feeding, we did not demonstrate hepatic folate deficiency per se, so it is possible that the response to dietary folate compensated for betaine depletion, or vice versa. Regardless, we can still make the conclusion that dietary betaine is as effective as folate at enhancing remethylation and transmethylation. Because the two remethylation pathways interact, more work is needed on respective enzyme regulation and potential therapies using both folate and betaine.

This study demonstrates an equal capacity of betaine and folate to enhance remethylation and transmethylation. Betaine was as effective as folate at lowering plasma homocysteine, which may represent a valuable strategy to overcome an underlying polymorphism affecting folate-mediated remethylation. Given betaine's ability to drive remethylation *via* both remethylation pathways, it may be theoretically more effective than folate at performing remethylation and lowering homocysteine concentrations. Furthermore, due to widespread polymorphisms in folate metabolism, the inclusion of betaine to infant formula, and multivitamin mixtures may be prudent.

The critical nature of transmethylation cannot be understated as a major consumer of methionine, and the metabolic capacity to fulfill this process is integral to the pediatric methionine requirement and essential for healthy development. Our nutritional model was designed to manipulate the metabolic pathways of the methionine cycle in order to quantify the contributions of dietary nutrients on remethylation and methionine availability. The availabilities of most methionine cycle nutrients (e.g. methionine, cysteine, betaine, choline, folate, vitamin B_6, vitamin B_{12}, creatine, phosphatidylcholine) are widely variable in infant diets and can impact methionine availability [25]. By manipulating some of these nutrients in our model, we were able to quantify the amount of available methionine generated from dietary betaine and folate. Such information can be used to refine neonatal methionine requirements; for example, the inclusion of folate and betaine in infant diets can furnish enough methionine for the transmethylation requirement during protein restriction in the neonate.

Acknowledgments and author contributions

JLR, LEM, JAB and RFB designed research. JLR and RFB wrote paper and have primary responsibility for final content. JLR, LEM conducted research. JLR, LEM, SVH, EWR analyzed data. All authors have read and approved the final manuscript.

References

[1] Robinson JL, Bartlett RK, Harding SV, Randell EW, Brunton JA, Bertolo RF. Dietary methyl donors affect in vivo methionine partitioning between transmethylation and protein synthesis in the neonatal piglet. Amino Acids 2016. doi:10.1007/s00726-016-2317-x.

[2] Robinson JL, Harding SV, Brunton JA, Bertolo RF. Dietary methyl donors contribute to whole-body protein turnover and protein synthesis in skeletal muscle and the jejunum in neonatal piglets. J Nutr 2016;146:2007–12. doi:10.3945/jn.115.226035.

[3] Varela-Moreiras G, Selhub J, Da Costa KA. Effect of chronic choline deficiency in rats on liver folate content and distribution. J Nutr Biochem 1992;3:519–22.

[4] Kim YI, Miller JW, Da Costa KA, Nadeau M, Smith D, Selhub J, et al. Severe folate deficiency causes secondary depletion of choline and phosphocholine in rat liver. J Nutr 1994;124:2197–203.

[5] Stead LM, Brosnan JT, Brosnan ME, Vance DE, Jacobs RL. Is it time to reevaluate methyl balance in humans? Am J Clin Nutr 2006;83:5–10.

[6] Pillai PB, Fanatico AC, Beers KW, Blair ME, Emmert JL. Homocysteine remethylation in young broilers fed varying levels of methionine, choline, and betaine. Poult Sci 2006;85:90–5.

[7] Davis SR, Quinlivan EP, Shelnutt KP, Ghandour H, Capdevila A, Coats BS, et al. Homocysteine synthesis is elevated but total remethylation is unchanged by the methylenetetrahydrofolate reductase 677C->T polymorphism and by dietary folate restriction in young women. J Nutr 2005;135:1045–50.

[8] Storch KJ, Wagner DA, Young VR. Methionine kinetics in adult men: effects of dietary betaine on L-[2H3-methyl-1-13C]methionine. Am J Clin Nutr 1991;54:386–94.

[9] Steenge GR, Verhoef P, Katan MB. Betaine supplementation lowers plasma homocysteine in healthy men and women. J Nutr 2003;133:1291–5.

[10] Robinson JL, McBreairty LE, Randell EW, Brunton JA, Bertolo RF. Restriction of dietary methyl donors limits methionine availability and affects the partitioning of dietary methionine for creatine and phosphatidylcholine synthesis in the neonatal piglet. J Nutr Biochem 2016;35:81–6. doi:10.1016/j.jnutbio.2016.07.001.

[11] McBreairty LE, Robinson JL, Harding SV, Randell EW, Brunton JA, Bertolo RF. Betaine is as effective as folate at re-synthesizing methionine for protein synthesis during moderate methionine deficiency in piglets. Eur J Nutr 2015. doi:10.1007/s00394-015-1049-0.

[12] Shoveller AK, Brunton JA, Pencharz PB, Ball RO. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J Nutr 2003;133:1390–7.

[13] Bidlingmeyer BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using pre-column derivatization. J Chromatogr 1984;336:93–104.

[14] Holm PI, Ueland PM, Kvalheim G, Lien EA. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Clin Chem 2003;49:286–94.

[15] MacKay DS, Brophy JD, McBreairty LE, McGowan RA, Bertolo RF.

Intrauterine growth restriction leads to changes in sulfur amino acid metabolism, but not global DNA methylation, in Yucatan miniature piglets. J Nutr Biochem 2012;23:1121–7. doi:10.1016/j.jnutbio.2011.06.005.

[16] Storch KJ, Wagner DA, Burke JF, Young VR. Quantitative study in vivo of methionine cycle in humans using [methyl-2H3]- and [1-13C]methionine. Am J Physiol 1988;255:E322–31.

[17] MacCoss MJ, Fukagawa NK, Matthews DE. Measurement of intracellular sulfur amino acid metabolism in humans. Am J Physiol Endocrinol Metab 2001;280:E947–55.

[18] Brouwer IA, van Dusseldorp M, Thomas CM, Duran M, Hautvast JG, Eskes TK, et al. Low-dose folic acid supplementation decreases plasma homocysteine concentrations: a randomized trial. Am J Clin Nutr 1999;69:99–104.

[19] Mudd SH, Poole JR. Labile methyl balances for normal humans on various dietary regimens. Metab Clin Exp 1975;24:721–35.

[20] Shoveller AK, Brunton JA, House JD, Pencharz PB, Ball RO. Dietary cysteine reduces the methionine requirement by an equal proportion in both parenterally and enterally fed piglets. J Nutr 2003;133:4215–24.

[21] Schubert HL, Blumenthal RM, Cheng X. Many paths to methyltransfer: a chronicle of convergence. Trends Biochem Sci 2003;28:329–35. doi:10.1016/S0968-0004(03)00090-2.

[22] Walkey CJ, Yu L, Agellon LB, Vance DE. Biochemical and evolutionary significance of phospholipid methylation. J Biol Chem 1998;273:27043–6. doi:10.1074/jbc.273.42.27043.

[23] Finkelstein JD, Kyle W, Harris BJ. Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue. Arch Biochem Biophys 1971;146:84–92.

[24] Finkelstein JD, Mudd SH. Trans-sulfuration in mammals. The methionine-sparing effect of cystine. J Biol Chem 1967;242:873–80.

[25] Robinson JL, Bertolo RF. The pediatric methionine requirement should incorporate remethylation potential and transmethylation demands. Adv Nutr 2016;7:523-34.

Figure Legends

Figure 1. Experimental design. Piglets were fed adequate methionine (Met) for 5 d followed by methionine restriction for 2 d. An intragastric infusion of methionine tracers assessed methionine kinetics in methyl-deplete piglets (MD-). Piglets were randomly grouped and rescued for 2 d with either folate (MD+F), betaine (MD+B) or both folate and betaine (MD+FB). Tracer kinetics were reassessed 2 d later; tissues were collected immediately after the second methionine infusion.

Figure 2. Time course data of the [^{13}C]methionine, [^{2}H]methionine infusions in plasma including subsequent labelling of [^{13}C]homocysteine and [^{2}H]choline. Plasma enrichments are expressed as mole percent excess (MPE) on the left axis and breath enrichment is expressed as atom percent excess (APE) on the right axis. Error bars indicate standard deviation among all piglets measured (n = 18).

Figure 3. Effects of dietary remethylation precursors on whole body transmethylation (A), remethylation (B), protein synthesis (C) and protein breakdown (D). Bars represent mean + SD for n = 6 piglets fed methyl-deplete diets for 7 d (Pre-Rescue) and rescued for 2 d (Post-Rescue) with either folate (MD+F), betaine (MD+B), or both folate and betaine (MD+FB). * indicates a significant main effect ($P < 0.05$) of Rescue with dietary remethylation precursors from 2-way ANOVA.

Table 1 Plasma concentrations of relevant metabolites at baseline[1] and after 7 d (Pre-Rescue) of dietary methyl restriction in piglets[2]

	Baseline	Pre-Rescue	*P*-value
Folate (ng/mL)	45.7 ± 22.0	16.1 ± 7.0	< 0.0001
Betaine (µM)	48.7 ± 27.0	<0.55	#
Choline (µM)	23.2 ± 14.00	6.9 ± 6.8	0.0001
DMG (µM)	9.73 ± 6.4	0.10 ± 0.16	< 0.0001
Methionine (µM)	16.0 ± 3.5	11.4 ± 3.5	0.0004
Homocysteine (µM)	28.8 ± 14.0	30.0 ±14.0	ns
Cysteine (µM)	58.8 ± 12.0	31.8 ± 7.0	< 0.0001

[1] Baseline data were from study Day-0 for plasma concentrations of folate, betaine, choline and dimethylglycine (DMG) and from Day-1 for plasma methionine, homocysteine and cysteine concentrations.
[2] Data represent pooled mean ± SD for n = 18 piglets tested by paired t-test.
 # indicates that betaine values were not compared statistically. 'ns' indicates not significant (*P* > 0.05)

Table 2 Plasma metabolites Pre-Rescue (Day-7) and Post-Rescue (Day-10) with dietary remethylation precursors[1]

	MD+F		MD+B		MD+FB	
	Pre-Rescue	Post-Rescue	Pre-Rescue	Post-Rescue	Pre-Rescue	Post-Rescue
Folate (nM)	33.2 ± 20.4	>181.8	34.7 ± 22.7	90.4 ± 54.5	41.8 ± 6.8	>181.8
Serine (μM)	70.1 ± 28.1	90.2 ± 62.9	86.7 ± 14.3	56.1 ± 14.8	84.0 ± 17.7	57.1 ± 31.2
Betaine (μM)	<0.55	<0.55	<0.55	146.0 ± 58.0	<0.55	103.0 ± 64.0
Choline (μM)	5.5 ± 4.7	1.9 ± 2.1	9.5 ± 9.7	5.8 ± 9.3	5.5 ± 5.2	7.2 ± 6.3
DMG (μM)	0.13 ± 0.26[a]	0.07 ± 0.07[a]	0.11 ± 0.08[a]	8.4 ± 10.2[b]	0.08 ± 0.11[a]	5.9 ± 7.7[b]
Methionine (μM)	10.0 ± 3.0	12.0 ± 5.8	12.3 ± 3.6	6.7 ± 1.6[*]	11.9 ± 3.9	9.4 ± 4.6
Homocysteine (μM)	32.1 ± 22.0	19.9 ± 12.0[*]	26.6 ± 6.0	23.5 ± 12.0[*]	31.4 ± 11.0	19.4 ± 10.0[*]
Cysteine (μM)	31.1 ± 9.0	34.9 ± 9.0	32.8 ± 5.0	32.6 ± 9.0	31.3 ± 7.0	37.3 ± 7.0
Taurine (μM)	20.4 ± 3.9	24.4 ± 17.0	25.0 ± 7.7	14.5 ± 2.8	17.0 ± 9.7	16.8 ± 6.7
Arginine (μM)	13.5 ± 2.2	10.7 ± 6.7	13.7 ± 5.7	17.8 ± 16.7	12.8 ± 6.9	11.6 ± 6.7
Glycine (μM)	119.9 ± 46.3	97.2 ± 26.0[*]	152.9 ± 23.1	101.0 ± 30.5[*]	140.3 ± 23.0	123.7 ±38.4[*]

[1] Data are mean ± SD for n = 6 piglets fed methyl-deplete diets for 7 d (Pre-Rescue) and rescued for 2 d (Post-Rescue) with folate (MD+F), betaine (MD+B), or both folate and betaine (MD+FB). DMG, dimethylglycine. Data with different letter superscripts were different by two-way ANOVA with Bonferroni post-test; [*] Indicates significant main effect ($P < 0.05$) of Rescue with dietary remethylation precursors.

Table 3 Kinetic parameters of a [$^{13}C_1$]methionine and [$^{2}H_3$-methyl]methionine infusion administered Pre-Rescue (Day-7) and Post-Rescue (Day-10) with dietary remethylation precursors[1]

	MD+F		MD+B		MD+FB		Rescue Effect[2]
	Pre-Rescue	Post-Rescue	Pre-Rescue	Post-Rescue	Pre-Rescue	Post-Rescue	*P*-value
	μmol Met/(kg·h)						
Remethylation (RM)	8.5 ± 5.3	15.9 ± 8.3*	9.4 ± 4.2	14.1 ± 6.8*	7.9 ± 6.0	16.0 ± 6.0*	<0.0001
Transmethylation (TM)	9.0 ± 5.2	16.5 ± 8.6*	10.7 ± 5.6	14.8 ± 6.7*	9.0 ± 6.5	16.9 ± 5.9*	<0.0001
Transsulfuration (TS)	0.6 ± 0.6	0.6 ± 0.7	1.1 ± 1.7	0.6 ± 1.0	1.1 ± 1.2	0.8 ± 0.7	ns
Protein Synthesis (PS)	124.1 ± 16.7	119.1 ± 22.3	136.3 ± 30.6	131.5 ± 22.3	143.6 ± 26.3	131.5 ± 20.2	ns
Protein Breakdown	37.2 ± 16.8	32.2 ± 22.2*	62.1 ± 34.8	37.9 ± 25.4*	57.2 ± 27.5	45.0 ± 20.6*	0.04
Protein Deposition	86.9 ± 0.7	86.8 ± 0.7	74.2 ± 29.7	74.6 ± 30.1	86.4 ± 1.3	86.6 ±0.7	ns
Flux ($Q_{[2H3\text{-methyl}]Methionine}$)	133.1 ± 15.2	135.2 ± 28.0	152.0 ± 31.4	150.6 ± 32.1	152.6 ± 31.0	148.5 ± 23.8	ns
Flux ($Q_{[13C1]Methionine}$)	124.7 ± 16.9	119.2 ± 23.3	137.4 ± 31.0	131.6 ± 32.4	144.7 ± 27.4	132.4 ± 20.6	ns
	%						
RM/TM	86.8 ± 18.0	95.8 ± 4.3	93.0 ± 9.7	95.7 ± 6.8	86.2 ± 21.1	94.4 ± 4.3	ns
TS/TM	13.1 ± 18.0	4.2 ± 4.3	7.0 ± 9.7	4.3 ± 6.7	13.8 ± 21.1	5.6 ± 4.3	ns
$TM/Q_{[2H3\text{-methyl}]Methionine}$	6.9 ± 4.2	11.9 ± 4.8*	7.0 ± 3.4	10.3 ± 5.8*	5.6 ± 3.2	11.3 ± 3.1*	0.0002

$PS/Q_{[2H3\text{-methyl}]Methionine}$	93.0 ± 4.3	88.6 ± 5.1*	89.9 ± 8.4	86.6 ± 15.4*	94.3 ± 3.2	88.7 ± 3.1*	0.003
TM/PS	7.7 ± 5.0	13.7 ± 6.4*	7.6 ± 4.0	13.7 ± 7.0*	6.1 ± 3.6	13.0 ± 4.1*	<0.0001
$[^{13}C_1]$Methionine	9.6 ± 1.7	9.1 ± 1.0	7.6 ± 1.9	8.2 ± 2.1	7.3 ± 1.5	9.1 ± 0.8	ns
$[^{13}C_1]$Homocysteine	7.2 ± 1.4	7.6 ± 1.5	6.7 ± 1.4	7.1 ± 1.6	6.3 ± 1.0	6.8 ± 0.9	ns
$[^{2}H_3$-methyl]Methionine	9.0 ± 1.3	8.1 ± 0.7	7.1 ± 2.2	7.3 ± 2.1	7.0 ± 1.4	8.3 ± 1.0	ns
$^{13}CO_2$	0.09 ± 0.10	0.11 ± 0.13	0.14 ± 0.21	0.08 ± 0.14	0.12 ± 0.14	0.10 ± 0.11	ns
$[^{2}H_3$-methyl]Choline	0.17 ± 0.07	0.31 ± 0.09*	0.15 ± 0.06	0.30 ± 0.12*	0.20 ± 0.06	0.31 ± 0.12*	<0.0001
$FPF_{Met \rightarrow Choline}$[3]	1.8 ± 0.9	3.5 ± 1.0*	2.0 ± 2.5	4.0 ± 3.3*	2.7 ± 0.9	3.4 ± 1.1*	0.0007
$FNC_{Met \rightarrow Choline}$[4]	2.1 ± 2.4	4.1 ± 3.5*	2.1 ± 2.4	4.1 ± 3.5*	2.9 ± 0.9	3.7 ± 1.3*	0.001

[1] Data are mean ± SD for n = 6 piglets, except for RM and TM parameters where n = 5. MD+F, methyl-deplete pigs rescued with folate, MD+B, methyl-deplete pigs rescued with betaine, MD+FB methyl-deplete pigs rescued with folate and betaine.
[2] Main effect of Rescue with dietary remethylation precursors; * indicates significant main effect ($P < 0.05$) from two-way ANOVA; 'ns' indicates $P > 0.05$.
[3] FPF is fraction of choline flux from methionine.
[4] FNC is fractional net conversion of methionine to choline factored for the contribution of the tracer infusion.

Table 4 Hepatic activities[1] of methionine synthase (MSyn), betaine:homocysteine methyltransferase (BHMT) and cystathionine beta-synthase (CBS) in pigs rescued with dietary remethylation precursors[2]

	MD+F	MD+B	MD+FB
	nmol product/(min·mg protein)		
MSyn	0.15 ± 0.02	0.13 ± 0.02	0.14 ± 0.04
BHMT	0.15 ± 0.04	0.17 ± 0.12	0.22 ± 0.07
CBS	0.96 ± 0.24	1.37 ± 0.50	0.97 ± 0.21

[1] Product is methionine for MSyn and BHMT, and cystathionine for CBS.
[2] n = 6 for all groups. MD+F, methyl-deplete pigs rescued with folate, MD+B, methyl-deplete pigs rescued with betaine, MD+FB methyl-deplete pigs rescued with folate and betaine. Values are means ± SD, tested by one-way ANOVA.

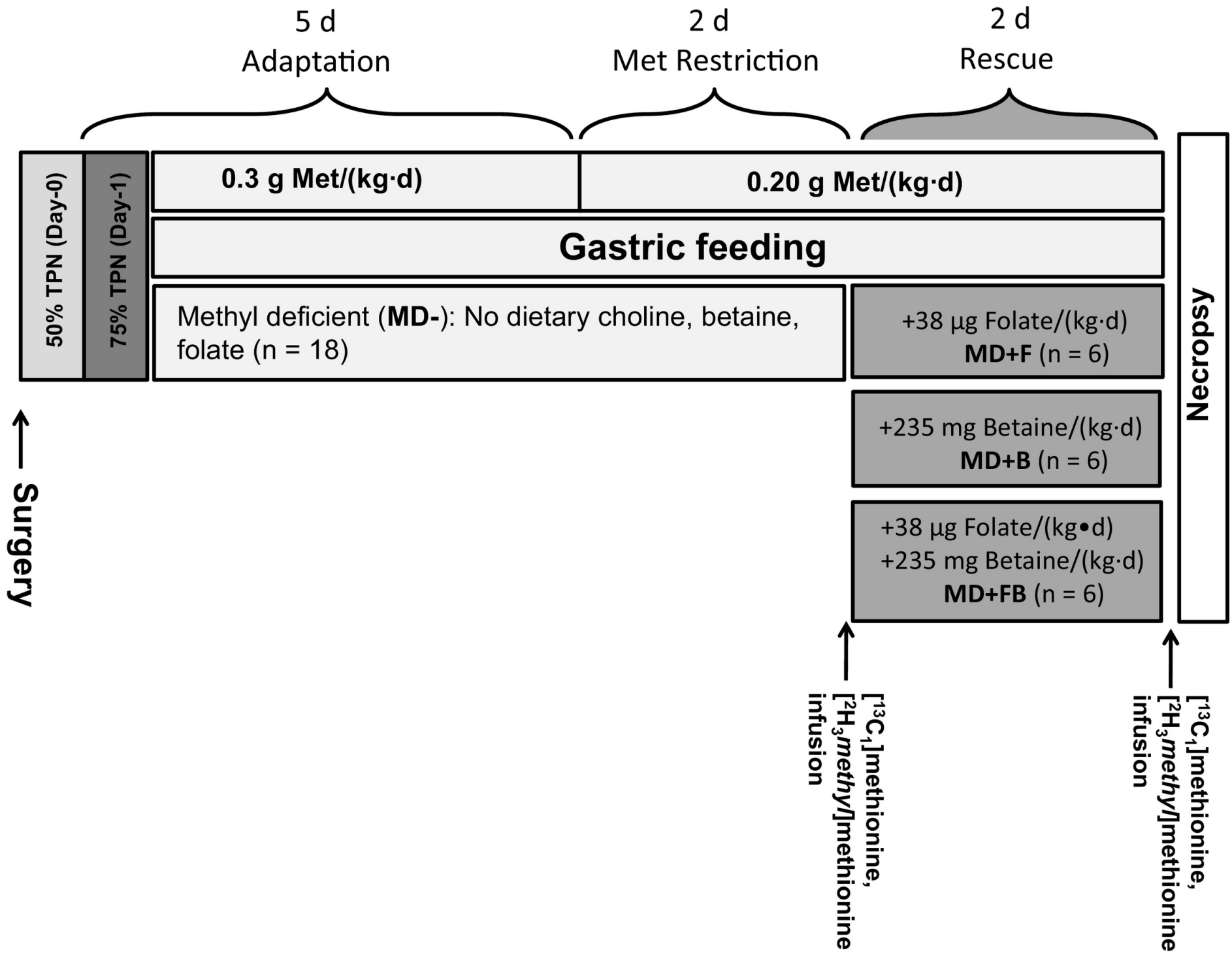
5 d
Adaptation
2 d
Met Restriction
2 d
Rescue
50% TPN (Day-0)
75% TPN (Day-1)
0.3 g Met/(kg·d)
0.20 g Met/(kg·d)
Gastric feeding
Methyl deficient (MD-): No dietary choline, betaine, folate (n = 18)
+38 µg Folate/(kg·d)
MD+F (n = 6)
+235 mg Betaine/(kg·d)
MD+B (n = 6)
+38 µg Folate/(kg•d)
+235 mg Betaine/(kg·d)
MD+FB (n = 6)
Necropsy
Surgery
[13C1]methionine, [2H3methyl]methionine infusion
[13C1]methionine, [2H3methyl]methionine infusion

Figure 1

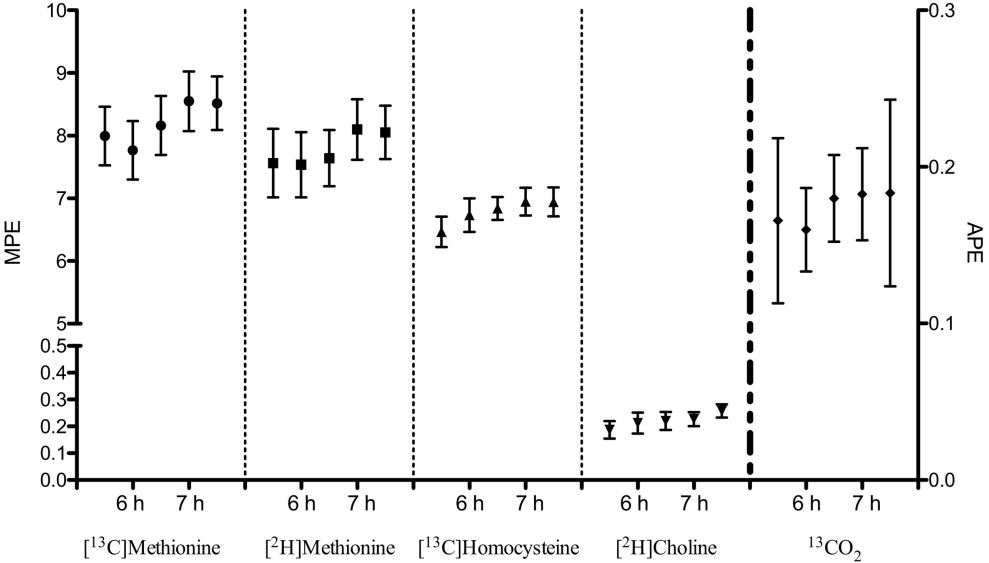
MPE
APE
10
9
8
7
6
5
0.5
0.4
0.3
0.2
0.1
0.0
0.3
0.2
0.1
0.0
6 h
7 h
[13C]Methionine
[2H]Methionine
[13C]Homocysteine
[2H]Choline
13CO2

Figure 2

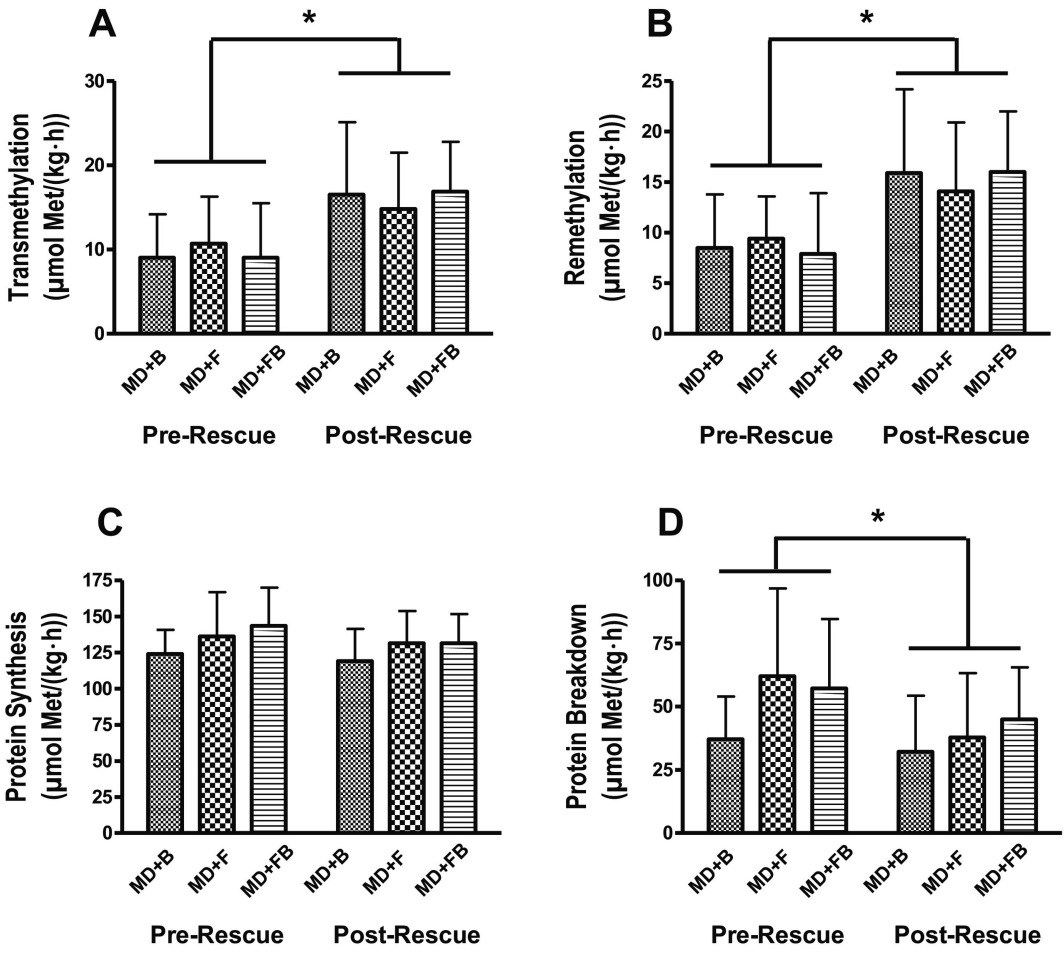
A
*
Transmethylation (µmol Met/(kg·h))
30
20
10
0
MD+B
MD+F
MD+FB
MD+B
MD+F
MD+FB
Pre-Rescue
Post-Rescue
B
*
Remethylation (µmol Met/(kg·h))
25
20
15
10
5
0
MD+B
MD+F
MD+FB
MD+B
MD+F
MD+FB
Pre-Rescue
Post-Rescue
C
Protein Synthesis (µmol Met/(kg·h))
175
150
125
100
75
50
25
0
MD+B
MD+F
MD+FB
MD+B
MD+F
MD+FB
Pre-Rescue
Post-Rescue
D
*
Protein Breakdown (µmol Met/(kg·h))
100
75
50
25
0
MD+B
MD+F
MD+FB
MD+B
MD+F
MD+FB
Pre-Rescue
Post-Rescue

Figure 3